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## Haemolytic activities and immunologic adjuvant effect of *Panax notoginseng* saponins

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**KEY WORDS** *Panax notoginseng*; saponins; haemolysis; immunologic adjuvant

### ABSTRACT

**AIM:** To evaluate haemolytic activities and adjuvant effect of *Panax notoginseng* saponins (PNS). **METHODS:** The haemolytic potential of PNS and its effects on the humoral and cellular immune responses to mice subcutaneously immunized with ovalbumin (OVA) were examined. ICR mice were immunized with OVA 100 µg alone, the mixture of OVA 100 µg and aluminoid 2 mg, or mixture of OVA 100 µg and PNS 50, 100, or 200 µg on the first and fifteenth day. Two weeks later (d 28), antigen-specific antibody in serum and concanavalin A (Con A)-, pokeweed (PWM)-, and phytohaemagglutinin (PHA)-stimulated splenocyte proliferation were investigated. **RESULTS:** Haemolytic percents of PNS-treated red blood cell were 11.6 % and 3.6 % at concentrations of 500 and 250 mg/L, respectively. Con A-, PWM- and PHA-stimulated splenocyte proliferation in the mice immunized with OVA/PNS (100 µg) was higher than that in the OVA control group ( $P < 0.01$ ). Mice immunized with OVA and PNS (50, 100, 200 µg) showed significantly higher serum OVA-specific IgG antibody titers ( $P < 0.01$ ) in comparison with the control group that received OVA alone. **CONCLUSION:** PNS possesses immunologic adjuvant activities and low-haemolytic effect.

### INTRODUCTION

New generations of vaccines, particularly those based on recombinant proteins and DNA, are likely to be less reactogenic than traditional vaccines, but are also less immunogenic. Therefore, there is an urgent need for the development of new and improved vaccine adjuvant<sup>[1,2]</sup>.

Saponins are chemically a heterogeneous group of sterol glycosides and triterpene glycosides that are

common constituents of plants. The triterpenoid saponins obtained from the bark of *Quillaja saponaria* Molina have been known to cause substantial enhancement of immune responses since the 1920s<sup>[3,4]</sup>. Quillaja saponins stimulate Th1 type immunity and are the only adjuvants reported to date that are capable of stimulating the production of antigen-specific cytotoxic T-lymphocyte (CTL) against exogenous or soluble protein antigens<sup>[5]</sup>. Although Quillaja saponins have these desirable adjuvant characteristics, their high toxicity, haemolytic effect, and instability have restricted their use in human vaccination<sup>[6-9]</sup>.

The present study was aimed to search for non-haemolytic saponins that could be possibly used as adjuvant in future large-scale studies of vaccination. Thus,

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the haemolytic activity of PNS and its effects on the humoral and cellular immune responses to mice subcutaneously immunized with ovalbumin (OVA) were examined.

## MATERIALS AND METHODS

**Mice** Male ICR mice (Grade II, 5 weeks old) weighing 18-22 g were purchased from Zhejiang Experimental Animal Center (Certificate No 22-2001001) and acclimatized for 1 week prior to use. Rodent laboratory chow and tap water were provided *ad libitum* and maintained under controlled conditions with a temperature of (24±1) °C, humidity of 50 %±10 %, and a 12-h light/12-h dark cycle.

**Chemicals** OVA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), pokeweed (PWM), phytohaemagglutinin (PHA), rabbit anti-mouse IgG peroxidase conjugate were purchased from Sigma; RPMI-1640 medium was from Gibco. Fetal Bovine Serum (FCS) was provided by Hangzhou Sijiqing Co; aluminum hydroxide gel (Alum) was from Zhejiang Wanma Pharm Co Ltd. All other chemicals were of AR grade.

**Preparation of PNS** Notoginseng Radix (root of *Panax notoginseng*) was purchased from drugstore in Hangzhou, and identified by the author according to the China Pharmacopoeia. Saponins were prepared as follows. Briefly, the powdered samples (1 kg) were extracted with 70 % EtOH at 100 °C (3×4 L), and then concentrated in vacuum (40 °C) to evaporate the solvent to give a small volume. After extracting with ether (3×0.5 L), the water layer portion was extracted with n-BuOH until n-BuOH layer became colorless. The n-BuOH solution was concentrated and dried in vacuum (60 °C). The dried extract was subjected to D101 resin column chromatography, washed with H<sub>2</sub>O, and eluted with EtOH to give PNS. PNS contained 61.8 %±1.2 % of notoginsenoside (including ginsenoside Rb<sub>1</sub>, Re, and Rg<sub>1</sub>) by thin layer chromatography and spectrophotometric method. PNS was dissolved in distilled water (pH 7.0) for later use.

**Haemolytic assay** Red blood cells were obtained from healthy New Zealand rabbit (Zhejiang Experimental Animal Center, China). Blood was collected with BD Vacutainer™ (NH 143 IU, Belliver Industrial Estate, Plymouth, UK). Aliquots of 7 mL of blood were washed three times with sterile saline solution (0.9 % w/v NaCl, pyrogen free) by centrifugation at 180×g for 5 min.

The cell suspension was prepared by finally diluting the pellet to 0.5 % in saline solution. A volume of 0.5 mL of the cell suspension was mixed with 0.5 mL diluent containing PNS 5, 10, 25, 50, 100, 250, 500, or 1000 mg/L in saline solution. The mixtures were incubated at 37 °C for 30 min, and centrifuged at 70×g for 10 min. Free haemoglobin in the supernatants was measured spectrophotometrically at 412 nm<sup>[11]</sup>. Saline and distilled water were used as minimal and maximal haemolytic controls. The haemolytic percent developed by the saline control was subtracted from all groups.

**Immunization** Animals were immunized subcutaneously with OVA 100 µg, the mixture of OVA 100 µg and Alum 2 mg, or the mixture of OVA 100 µg with PNS 50, 100, or 200 µg, in 0.2 mL saline solution. Saline-treated animals were used as controls. A boosting injection was given 2 weeks later. Sera and splenocytes were collected 2 weeks after the second immunization for proliferation assay and measurement of OVA-specific antibody.

**Splenocyte proliferation assay** Single cell suspensions were prepared by grinding the spleen sample gently against a stainless-steel mesh in cold RPMI-1640 under aseptic condition and the erythrocytes were lysed with ammonium chloride (0.8 %, w/v). After a centrifugation (380×g at 4 °C for 10 min), the pelleted cells were washed and resuspended in complete medium [RPMI-1640 supplemented with HEPES 12 mmol/L (pH 7.1), 2-mercaptoethanol 0.05 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L, and 10 % FCS]. The number of cells was counted with a haemocytometer following staining with Trypan blue. Cell viability exceeded 95 %. Splenocyte proliferation was assayed as previously described<sup>[10]</sup>. Briefly, Splenocytes were seeded into 4-5 wells of a 96-well flat-bottom microtiter plate (Nunc) at a cell density of 1×10<sup>10</sup>/L in 100 µL complete medium where Con A (final concentration 5 mg/L), PWM (final concentration 5 mg/L), PHA (final concentration 10 mg/L), or medium were added to a final volume of 200 µL. The plate was incubated at 37 °C in a humid atmosphere with 5 % CO<sub>2</sub>. After 68 h, 50 µL of MTT solution (2 g/L) was added to each well and incubated for 4 h. The microtiter plates were centrifuged (1400×g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well 200 µL of Me<sub>2</sub>SO working solution (180 µL Me<sub>2</sub>SO with 20 µL HCl 1 mol/L) was added, and the absorbance (*A*) was evaluated in an ELISA reader at 570 nm with a 630 nm reference after 15 min. The Stimulation index (SI)

was calculated by the formula:

$$SI = \frac{A \text{ value of cell culture with a mitogen}}{A \text{ value of cell without a mitogen}}$$

**Measurement of OVA-specific antibody** OVA-specific IgG antibody in serum was measured by an indirect ELISA. In brief, microtiter plate wells (Nunc) were coated with OVA (6.25 mg/L in carbonate-bicarbonate buffer 50 mmol/L, pH 9.6) at 4 °C for 24 h. The wells were washed three times with PBS containing 0.05 % (v/v) Tween-20 (PBS/Tween), and then blocked with 1 % FCS/PBS at 37 °C for 1 h. After 3 times of washing, 100 µL of 1:400 diluted serum sample was added to triplicate wells. The plates were then incubated at 37 °C for 1 h, followed by 3 times of washing. Aliquots of 100 µL of rabbit anti-mouse IgG horseradish peroxidase conjugate diluted with BSA/Tween were added to each plate. The plates were further incubated at 37 °C for 1 h. After washing, the peroxidase activity was assayed as follows: 100 µL of substrate solution (10 mg of *o*-phenylene-diamine and 8 µL of 30 % H<sub>2</sub>O<sub>2</sub> in 25 mL of citrate-phosphate buffer 0.1 mol/L, pH 5.0) was added to each well. The plate was incubated for 10 min at 37 °C, and enzyme reaction was terminated by adding 50 µL per well of H<sub>2</sub>SO<sub>4</sub> 1 mol/L. The absorbance was measured in an ELISA reader at 490 nm with a 595 nm reference.

**Statistical analysis** The data were expressed as mean±SD and examined for their statistical significant difference with analysis of variance.

## RESULTS

**Haemolytic activities** Haemolytic percents of red blood cell treated with PNS 500 mg/L and 250 mg/L were 11.6 % ( $P < 0.05$ ) and 3.6 % ( $P > 0.05$ ), respectively. PNS showed no haemolytic activity at concentrations of 2.5-250 mg/L (Tab 1).

**Effect of PNS on mitogen-stimulated splenocyte proliferation** Con A- and PHA-stimulated splenocyte proliferation in the mice immunized with OVA/PNS (100, 200 µg) was higher than that in the OVA control group ( $P < 0.01$ ). Splenocytes isolated from OVA/PNS (100 µg)-immunized mice and stimulated by PWM showed a greater proliferative response than that of OVA alone group ( $P < 0.01$ ). However, no significant difference ( $P > 0.05$ ) was observed between the OVA group and OVA/Alum group (Fig 1).

**Tab 1. Haemolytic activities of *Panax notoginseng* saponins (PNS). Haemolytic percents of saline and distilled water were used as minimal and maximal haemolytic controls.  $n=3$  tests. Mean±SD. <sup>a</sup> $P > 0.05$ , <sup>b</sup> $P < 0.05$  vs saline group.**

Group	Absorbance value	Haemolytic percent/%
Distilled water	1.64±0.04	100.0±2.4
Saline	0.094±0.009	0.0±5.2
PNS 500 mg·L <sup>-1</sup>	0.284±0.019	11.6±1.4 <sup>b</sup>
250	0.153±0.007	3.6±1.3 <sup>a</sup>
125	0.090±0.015	-0.22±0.55
50	0.072±0.009	-1.3±0.7
25	0.073±0.011	-1.3±0.9
12.5	0.078±0.007	-1.0±0.5
2.5	0.061±0.005	-2.0±0.4

**Effect of PNS on the OVA-specific serum IgG antibody response** Mice immunized with OVA and Alum (2 mg) or PNS (50, 100, 200 µg) showed higher serum antibody titers than that of OVA alone group ( $P < 0.01$ ). Moreover, There was no significant difference between the OVA with PNS (100 µg) group and OVA with Alum group (Fig 2).

## DISCUSSION

The unique capacity of Quillaja saponins to stimulate both the Th1 immune response and the production of cytotoxic T-lymphocyte (CTL) against exogenous antigens makes them ideal for use in subunit vaccines and vaccines directed against intracellular pathogens as well as for therapeutic cancer vaccines<sup>[5]</sup>. In fact, there are a series of commercial veterinary vaccines as well as human vaccines formulated with this kind of adjuvant undergoing clinical evaluation. QS-21 has been evaluated in a large number of vaccines in Phase I and Phase II human clinical trials<sup>[11]</sup>. These vaccines include cancer immunotherapeutics<sup>[12,13]</sup>, HIV recombinant envelope<sup>[14,15]</sup>, and malarial antigens<sup>[16]</sup>. However, Quillaja saponins have serious drawbacks. For instance, they are not stable in aqueous phase because of hydrolysis of their ester moieties on the C-28 fucose<sup>[7-9]</sup>. In contrast to the majority of saponins from other species, Quillaja saponins are acylated. The three most predominant saponins (QS-17, QS-18, and QS-21) are acylated at the 4-hydroxyl position of fucose with two

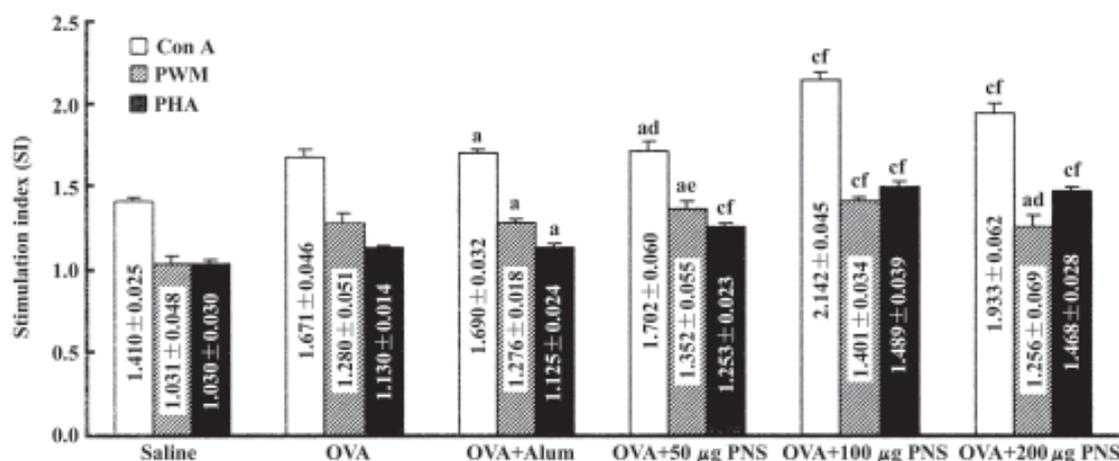


Fig 1. Effect of *Panax notoginseng* saponins (PNS) on mitogen-stimulated splenocyte proliferation. ICR mice were immunized sc with OVA 100 µg alone or with OVA 100 µg dissolved in saline containing Alum 2 mg or PNS (50, 100, or 200 µg) on the first and fifteenth day. Splenocytes were prepared 2 weeks after the last immunization and cultured with Con A, PWM, or PHA for 72 h. Splenocyte proliferation was measured by MTT.  $n=5$  mice. Mean±SD. <sup>a</sup> $P>0.05$ , <sup>c</sup> $P<0.01$  vs OVA group. <sup>d</sup> $P>0.05$ , <sup>e</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  vs OVA+Alum group.

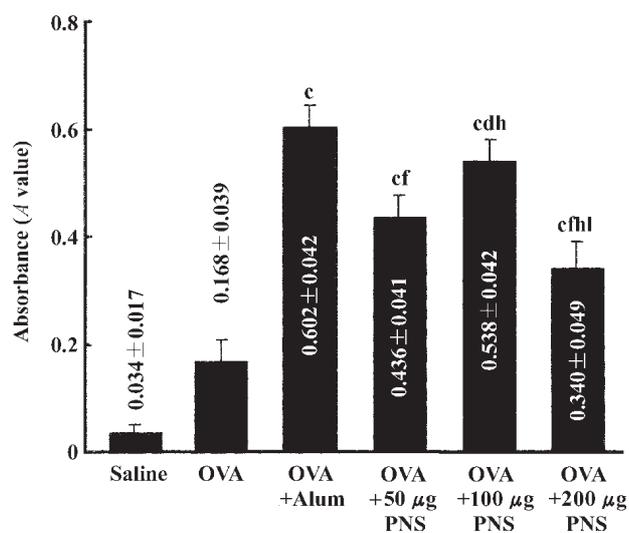


Fig 2. Effect of *Panax notoginseng* saponins (PNS) on OVA-specific IgG antibody. ICR mice were immunized sc with OVA 100 µg alone or with OVA 100 µg dissolved in saline containing Alum (2 mg) or PNS (50, 100, or 200 µg) on the first and fifteenth day. Sera were collected 2 weeks after the last immunization. OVA-specific IgG antibodies in the sera were measured by an indirect ELISA.  $n=5$  mice. Mean±SD. <sup>c</sup> $P<0.01$  vs OVA group. <sup>d</sup> $P>0.05$ , <sup>e</sup> $P<0.01$  vs OVA+Alum group. <sup>h</sup> $P<0.05$  vs OVA+PNS (50 µg) group. <sup>i</sup> $P<0.01$  vs OVA+PNS (100 µg) group.

linked 3,5-dihydroxy-6-methyloctanoic acids containing a glycosylation site at the 5-OH position of the acyl chains. The acylation is highly critical to Th1 type re-

sponse and the production of CTL<sup>[9]</sup>. These saponins stored under mild condition are deacylated. Deacylated saponins may lose both their capacity to stimulate the Th1 immune response and to produce antigen-specific CTL, but stimulate the Th2 immune response instead. Another disadvantage of Quillaja saponins that limits their use as adjuvant is their high toxicity and tendency to induce tissue damage. The latter is at least partly associated with their haemolytic activity<sup>[6]</sup>. The Quil A is lethal to mice in the dose range of 100-125 µg, while QS-21 is lethal only at 500 µg. Saponins QS-17, 18, and 21 caused 50 % haemolysis at the concentrations of 25, 15, and 7 mg/L, respectively<sup>[3]</sup>.

To circumvent these obstacles, non-toxic, non-haemolytic, and stable saponins have to be selected fulfilling certain criteria. Therefore, PNS was screened in this investigation for the haemolytic activity on rabbit erythrocytes. We found that PNS showed a slight haemolytic effect, with 11.6 % and 3.6 % at concentrations of 500 and 250 mg/L, respectively. LD<sub>50</sub> (ip) of PNS to mice was 825.6 (730.7-934.8) mg/kg<sup>[17]</sup>. Thus, PNS was safer than Quil A and its components in clinical use.

We also evaluated whether PNS could enhance the humoral and cellular immune responses to OVA in mice when given together with OVA. Our results showed that PNS enhanced significantly not only the specific IgG antibody response, but also the mitogen-stimulated splenocyte proliferation. In conclusion, PNS possesses

immunologic adjuvant activities and low-haemolytic effect.

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